# AD-A218 725

## UMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. unclassified

1b. RESTRICTIVE MARKINGS

2a. SECURITY CLASSIFICATION AUTHORITY

2b. DECLASSIFICATION / DOWNGRADING SCHEDULE

3. DISTRIBUTION / AVAILABILITY OF REPORT
Approved for public release; distribution is unlimited.

4. PERFORMING ORGANIZATION REPORT NUMBER(S)
USAFSAM-JA-89-58

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION
USAF School of Aerospace Medicine

6b. OFFICE SYMBOL (If applicable)
USAFSAM/NGNS

7a. NAME OF MONITORING ORGANIZATION

6c. ADDRESS (City, State, and ZIP Code)
Human Systems Division (AFSC)
Brooks Air Force Base, TX 78235-5301

7b. ADDRESS (City, State, and ZIP Code)

8a. NAME OF FUNDING / SPONSORING ORGANIZATION
Air Force Office of Scientific Research

8b. OFFICE SYMBOL (If applicable)
AFOSR/NL

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (City, State, and ZIP Code)
Air Force Office of Scientific Research(AFSC)
Bolling Air Force Base, DC 20332-6448

10. SOURCE OF FUNDING NUMBERS

| PROGRAM ELEMENT NO. | PROJECT NO. | TASK NO | WORK UNIT ACCESSION NO. |
|---|---|---|---|
| 61102F | 2312 | W6 | 10 |

11. TITLE (Include Security Classification)
Light Increases Fors-related Protein Immunoreactivity in the Rat Suprachiasmatic Nuclei

12. PERSONAL AUTHOR(S)
Rea, Michael A.

| 13a. TYPE OF REPORT | 13b. TIME COVERED | 14. DATE OF REPORT (Year, Month, Day) | 15. PAGE COUNT |
|---|---|---|---|
| Final | FROM 89/04 TO 89/08 | 1989, August 29 | 21 |

16. SUPPLEMENTARY NOTATION
Animal use Protocol #NGNS-87-20

| 17. COSATI CODES | | |
|---|---|---|
| FIELD | GROUP | SUB-GROUP |
| 06 | 04 | |
| | | |

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
C-fos, Suprachiasmatic Nuclei, Light, Entrainment, Rat

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Fifteen minutes of bright, white light exposure at mid subjective night resulted in a marked increase in both the number and stain density of c-fos protein (Fos)-immunoreactive neurons in the suprachiasmatic nuclei (SCN). In all cells, peroxidase reaction product was confined to the nucleus. Most Fos-immunoreactive cells were concentrated in the ventrolateral third of the SCN, although a few immunoreactive cells were also observed diffusely distributed along the dorsal border of the nucleus and into the surrounding hypothalamus. Along the rostro-caudal extent of the SCN, the greatest density of Fos-immunoreactive cells was found at a level approximately 300 - 400 m caudal of the rostral pole of the nucleus. The population of Fos-immunoreactive cells in the SCN lies within the terminal fields of both the retinohypothalamic and geniculohypothalamic tracts. In addition, a few Fos-immunoareactive cells were observed in the ventral lateral geniculate nucleus. The results suggest that retinal illumination induces transsynaptic c-fos expression in a select population of SCN neurons.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION
Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL
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22c. OFFICE SYMBOL
USAFSAM/NGNS

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

# Light increases Fos-related protein immunoreactivity in the rat suprachiasmatic nuclei

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Running Title: Fos immunoreactivity in the SCN

Key Words: c-fos, suprachiasmatic nuclei, light, entrainment, rat, retinal afferents



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Footnote: The animals involved in this study were procured, maintained and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources - National Research Council.

ABSTRACT

REA, M. A., Light increases Fos-related protein immunoreactivity in the rat suprachiasmatic nuclei. BRAIN RES BULL. Fifteen minutes of bright, white light exposure at mid subjective night resulted in a marked increase in both the number and stain density of c-fos protein (Fos)-immunoreactive neurons in the suprachiasmatic nuclei (SCN). In all cells, peroxidase reaction product was confined to the nucleus. Most Fos-immunoreactive cells were concentrated in the ventrolateral third of the SCN, although a few immunoreactive cells were also observed diffusely distributed along the dorsal border of the nucleus and into the surrounding hypothalamus. Along the rostro-caudal extent of the SCN, the greatest density of Fos-immunoreactive cells was found at a level approximately 300 - 400 μm caudal of the rostral pole of the nucleus. The population of Fos-immunoreactive cells in the SCN lies within the terminal fields of both the retinohypothalamic and geniculohypothalamic tracts. In addition, a few Fos-immunoreactive cells were observed in the ventral lateral geniculate nucleus. The results suggest that retinal illumination induces transsynaptic c-fos expression in a select population of SCN neurons.

INTRODUCTION

A light-entrainable pacemaker located in the suprachiasmatic nuclei (SCN) appears to be responsible for the generation of a wide range of circadian physiological and behavioral rhythms in mammals (41). Bilateral destruction or surgical isolation of the SCN abolishes circadian rhythmicity in rodents (23,25,41), and transplantation of fetal SCN tissue into the third ventricle of SCN-lesioned hosts restores rhythmicity (8,31,45). Furthermore, circadian rhythms in single unit activity (15,17), 2-deoxyglucose utilization (37), and vasopressin release (13,15) persist in the isolated SCN in vitro indicating that rhythm generation is intrinsic to the nucleus.

Circadian rhythms, which persist under constant environmental conditions (free running), are normally entrained to the external light-dark (LD) cycle. In rodents maintained under constant darkness, the free-running activity rhythm can be phase shifted by light pulses in a phase-dependent manner (7,47). Light pulses delivered early in the active period result in phase delays of the pacemaker, while pulses given late in the active period cause phase advances. This phase-dependent response to light is crucial to the photic entrainment process (41).

The SCN receive entraining photic information by at least two pathways: a monosynaptic projection from retinal ganglion cells (24,26,35,38,39), the retinohypothalamic tract (RHT), and an indirect retinal projection through the ventral lateral geniculate nucleus (vLGN)/intergeniculate leaflet (3,39,46), the geniculohypothalamic tract (GHT). The RHT projection appears to be both necessary and sufficient to support photic entrainment of

the pacemaker (24,25). The role of the GHT in photic control of the pacemaker is unclear. Although both the RHT and GHT projection fields have been described in some detail (26,38,46), little is known concerning the identity of SCN neurons responsive to photic stimulation or the neurochemical sequelae responsible for light-induced alterations of pacemaker activity.

The c-fos proto-oncogene codes for a nuclear phosphoprotein with DNA binding properties consistent with its proposed role as a transcription regulator (9,40,44). In the CNS, c-fos expression increases in response to chemically-induced seizure activity (11,30,36,42), as well as a variety of other physiological and pharmacological stimuli (10,12,27), suggesting that Fos may participate in the process of transsynaptic regulation of gene expression by neurotransmitters or neuromodulators (1,16). The observation that physiologically-relevant stimuli induce c-fos expression in appropriate CNS regions (21,43) led Sagar et al. (43) to propose the use of Fos immunocytochemistry as a technique for mapping physiologically-activated neurons in brain. Using this approach, we have identified a population of neurons in the rat SCN that accumulate Fos in response to retinal illumination.

METHODS

Male, Sprague-Dawley rats weighing 200-250 gm (Charles River) were housed 5 per cage under LD 12:12 (lights on at 0300; intensity was approximately 300 lux) for at least 3 weeks prior to experimentation. Rat chow and water were provided ad libitum. At 1500 (time of lights out) on the day before stimulation, rats were transferred to constant dim (<1 lux) red illumination. At

2100 (mid dark phase) on the following day, a group of rats (n=9) received a single 15 minute pulse of bright (2200 lux) white light and were returned to dim red illumination. Control rats (n=7) were transferred to the stimulation chamber but were not exposed to light. The light stimulation apparatus was a Vivitar Model 2000AF slide projector, consisting of a 150 W tungsten-halogen lamp (type A1/216), a glass infrared filter, and a series of collimating and projection lenses. Light intensity could be controlled by introducing 5 cm x 5 cm neutral density filters into the slide compartment. The stimulation chamber was a white styrofoam box (22 cm x 30 cm x 30 cm). The light source was suspended above the chamber such that the light intensity on the floor of the chamber was 2200 lux (Tektronics J-16 photometer with J6511 probe). Three hours after stimulation, rats were deeply anesthetized with ketamine (100 mg/g) and xylazine (4 mg/g) and perfused transcardially with 150 ml heparinized saline and 100 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) containing 15% (v/v) saturated picric acid. Brains were removed, cut into blocks and incubated at $4^{o}$ C in fixative for 16 hours, followed by an additional 24 hours in 20% sucrose in 0.1 M phosphate buffer (pH 7.4). Frozen blocks were cut in the coronal plane to a thickness of 50 µm. The sections were washed twice with phosphate buffer and incubated at room temperature in 0.1 M sodium phosphate buffer containing 0.2% Triton X-100, 0.1% bovine serum albumin and 2% normal goat serum (TPBG). Next, the sections were incubated for 42 hours at $4^{o}$ C in affinity-purified Fos antiserum (kindly provided by Dr. Tom Curran) diluted 1:100 in TPBG. This antiserum was raised against

synthetic M-peptide, corresponding to residues 127-152 of the Fos protein (5), and recognizes a number of Fos-related antigens (6,14). After incubation, sections were washed in phosphate buffer and processed with the Vectastain ABC Kit (Vector Labs) using diaminobenzidine as a chromagen (43). Selected sections were lightly stained with cresyl violet and eosin. Control incubations were performed using sections from stimulated rats incubated in the presence of 10 µg/ml of synthetic M-peptide in 1:100 Fos antiserum.

RESULTS

Fifteen minutes of bright light exposure caused a dramatic increase in Fos immunoreactivity (Fos-ir) in the SCN (Fig.1B). Increases were observed in both the number and stain density of immunoreactive cells. In all cells, peroxidase reaction product was confined to the nucleus. Light-level microscopic examination of Fos-ir cells revealed morphology characteristic of neurons (not shown). However, the presence of Fos-ir neuroglia in the SCN could not be ruled out. Control sections incubated in the presence of synthetic M-peptide showed no nuclear stain.

In the SCN of light-exposed rats (n=9), Fos-ir cells were concentrated in the ventrolateral third of the nucleus. A few immunoreactive cells were also observed diffusely distributed along the dorsal border of the nucleus and into the surrounding hypothalamus (Fig. 2). Along the rostro-caudal extent of the SCN, the greatest density of Fos-ir cells was found at a level approximately 300 - 400 µm caudal of the rostral pole of the nucleus (Fig. 2). At this level, several immunoreactive neurons

were also observed embedded within the optic chiasm. Very few Fos-ir cells were present in the anterior hypothalamic area (AHA), the lateral hypothalamic area (LHA), or the retrochiasmatic area (RCA) in sections from either control or stimulated rats.

The number of Fos-ir cells present in the SCN of unstimulated rats (n=7) was quite variable. In sections from unstimulated animals that showed appreciable staining (Fig. 1A), the distribution of Fos-ir cells was similar to that observed after stimulation. However, in 3 separate experiments, the number of immunoreactive cells present in control sections never approached the quantity observed after light stimulation.

In addition to the SCN, other diencephalic and midbrain nuclei were inspected for Fos-immunoreactivity. Approximately 20% of neurons in the supraoptic nuclei (SON) showed low levels of Fos-ir in both control and stimulated rats (Fig 2.). Light stimulation did not appear to alter the number or distribution of immunoreactive cells in the SON. Since the hypothalamic paraventricular nuclei (PVN) appear to mediate the nocturnal rise in pineal melatonin production (28), and light exposure during the latter half of the dark phase suppresses melatonin production (29), this region was examined for c-fos expression as well. Again, a few scattered Fos-ir cells were observed in the PVN area but their number did not appear to change following light stimulation (data not shown). Finally, the geniculate complex and the superior colliculi (SC) were examined for evidence of light-induced c-fos expression (n=2). No Fos-ir cells were observed in the SC. However, a few (10-15 cells/section) immunoreactive

neurons were observed scattered within the ventral lateral geniculate nucleus (vLGN) of light-stimulated rats (not shown).

## DISCUSSION

The results of the present study reveal a population of SCN neurons that accumulate Fos in response to retinal illumination. As appears to be the case elsewhere in brain (10,12,36), Fos accumulation probably reflects an increase in c-fos gene expression in immunoreactive cells. Although we have not yet confirmed this possibility by directly measuring c-fos mRNA accumulation, this report may represent the first demonstration of transsynaptic induction of gene expression in response to acute stimulation with light. An elucidation of the mechanism by which light stimulation induces c-fos expression in SCN neurons would contribute to our understanding of the neurochemistry of photic entrainment and, possibly, provide insight into the time-keeping mechanism of the SCN pacemaker.

Although light exposure consistently produced increases in Fos expression in the SCN, Fos-ir cells were always observed in sections from unstimulated rats as well. Perhaps some fos expression occurs in SCN neurons in the absence of photic stimulation. Alternatively, non-photic activation of Fos-ir neurons may have occurred, possibly as a consequence of handling. It is also possible that the dim red illumination employed before and after transfer to the stimulation chamber was sufficient to induce fos expression. McCormack and Sontag (32) have reported that red (>600 nm) illumination at intensities well below those employed in the current study were sufficient to influence pace-

maker function in albino rats.

The vast majority of cells that displayed Fos-ir in response to light stimulation were located in the ventrolateral SCN, well within the reported terminal fields of both the RHT (26,35) and GHT projections (3,46). However, the data available are insufficient to determine if SCN cells that accumulate Fos in response to retinal illumination receive RHT or GHT afferents. Very few immunoreactive cells were observed in regions of the AHA, LHA or RCA that have been reported to receive RHT innervation in the rat (26). Thus, not all neurons that receive direct retinal afferents make Fos in response to light exposure. This was clearly the case in the geniculate complex, where only a few neurons in the vLGN were immunoreactive for Fos (not shown). Perhaps retinoreceptive SCN neurons are unique in their response to RHT stimulation (33). Alternatively, GHT innervation might be necessary to support light-induced fos expression in the SCN. Indeed, the distribution of neuropeptide Y-containing GHT terminals in the SCN (4) is very similar to that reported here for Fos-ir cells. Additional experiments employing selective lesions of the GHT, or cuts which isolate the SCN while sparing RHT input, will be required to determine the extent to which the RHT and GHT contribute to light-induced fos expression.

Another important issue for future investigation is the neurochemical identity of Fos-ir cells in the SCN. VIP-containing cells, which are also concentrated in the ventrolateral SCN (2,48), appear to be likely candidates. Some VIP-immunoreactive cells appear to receive both NPY-containing GHT afferents (20) and/or direct RHT afferents (22). Since Fos immunostaining in

the SCN is restricted to cell nuclei, this issue can be easily resolved by combining the technique with other immunocytochemical procedures that label cytoplasmic or vesicular neurotransmitter markers or neuropeptides.

Finally, there is the question of the role, if any, that Fos-ir cells may play in SCN pacemaker function. Obviously, the fact that these cells respond in a novel way to retinal illumination suggests a possible involvement in the photic entrainment process. Future work to determine if fos expression is a necessary step in the response cascade leading to light-induced phase shifts of the pacemaker would address this possibility. Fos expression appears to be an early event in processes that result in long term alterations in cell function (1,16). In this regard, it is noteworthy that Gueldner and coworkers (18,19) have reported the occurrence of postsynaptic structural alterations in retinoreceptive SCN neurons in response to changes in environmental illumination. However, a more intriguing possibility is that Fos-ir cells are, in fact, cellular components of the SCN pacemaker. It should be pointed out that, in the present study, we chose to stimulate animals during the mid-subjective night, a time at which the pacemaker is sensitive to retinal illumination and light pulses cause phase advances of pacemaker-driven rhythms (7,47). Fos expression could represent an early evert in the phase response of SCN pacemaker cells. If this is the case, fos expression in response to photic stimulation should show phase-dependence similar to the reported for pacemaker-driven rhythms (7,47).

ACKNOWLEDGEMENTS

The author gratefully acknowledges the generosity of Dr. Tom Curran, Roche Institute of Molecular Biology, for providing Fos antiserum and Dr. David Earnest, University of Rochester, for providing synthetic M-peptide. Thanks to Dan Felder for technical support, Bob Burnes and Raymond Blancarte for photography and graphics, and Marjorie Bolt for assistance in preparing the manuscript. Supported by AFOSR 2312W6.

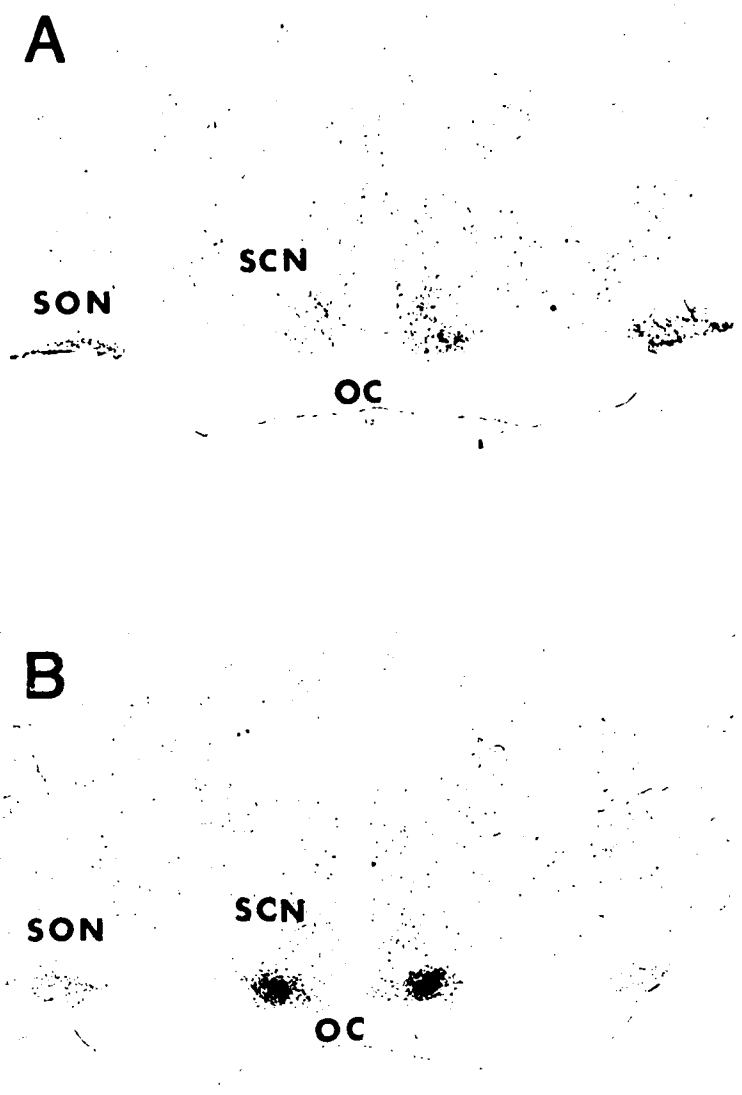


FIG 1. Representative photomicrographs of coronal sections through the suprachiasmatic hypothalamus of (A) an unstimulated control rat, and (B) a rat which received 15 minutes of bright (2200 lux) white light exposure 3 hours before fixation. Sections have been stained for Fos immunoreactivity as described in METHODS. SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; OC, optic chiasm. Magnification = 30X.

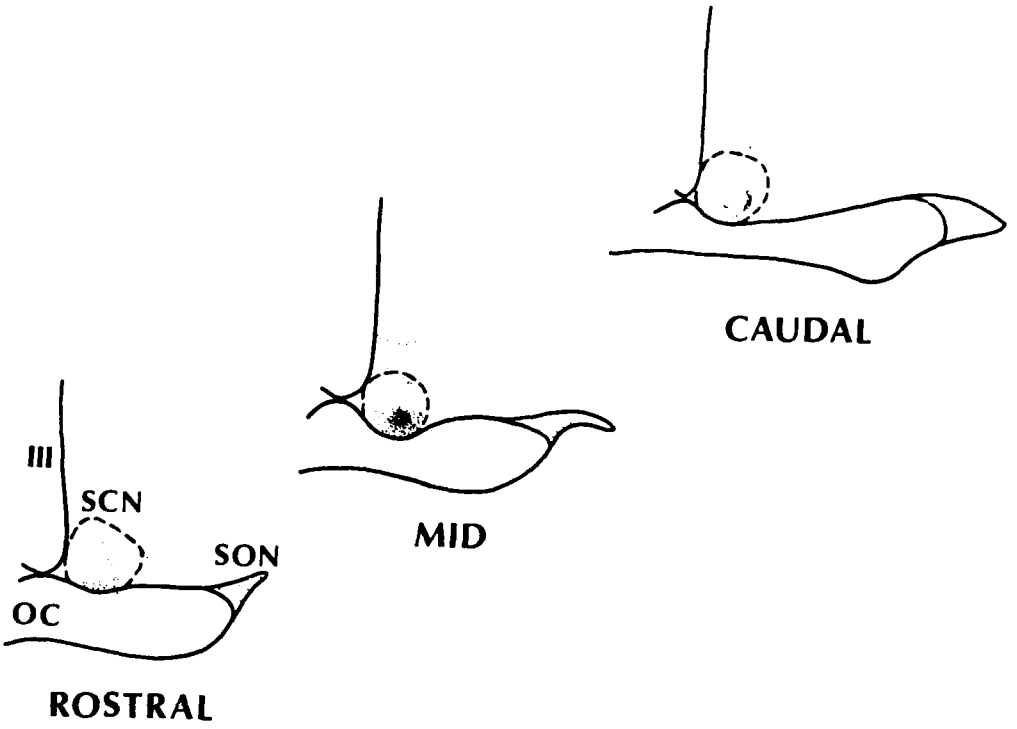


FIG 2. Distribution of Fos immunoreactivity at three levels along the rostro-caudal extent of the SCN. Dots denote immunoreactive nuclei as observed in representative sections from light-stimulated rats. SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; OC, optic chiasm; III, third ventricle.

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